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A point mutation of valine-311 to methionine in *Bacillus subtilis* protoporphyrinogen oxidase does not greatly increase resistance to the diphenyl ether herbicide oxyfluorfen

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Abstract

In an effort to asses the effect of Val311Met point mutation of *Bacillus subtilis* protoporphyrinogen oxidase on the resistance to diphenyl ether herbicides, a Val311Met point mutant of *B. subtilis* protoporphyrinogen oxidase was prepared, heterologously expressed in *Escherichia coli*, and the purified recombinant Val311Met mutant protoporphyrinogen oxidase was kinetically characterized. The mutant protoporphyrinogen oxidase showed very similar kinetic patterns to wild type protoporphyrinogen oxidase, with slightly decreased activity dependent on pH and the concentrations of NaCl, Tween 20, and imidazole. When oxyfluorfen was used as a competitive inhibitor, the Val311Met mutant protoporphyrinogen oxidase showed an increased inhibition constant about 1.5 times that of wild type protoporphyrinogen oxidase. The marginal increase of the inhibition constant indicates that the Val311Met point mutation in *B. subtilis* protoporphyrinogen oxidase may not be an important determinant in the mechanism that protects protoporphyrinogen oxidase against diphenyl ether herbicides.

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1. Introduction

Protoporphyrinogen oxidase (Protox, EC 1.3.3.4), the last common enzyme in the biosynthesis of hemes and chlorophylls [1], catalyzes the six-electron oxidation of protoporphyrinogen IX (Protogen IX) to protoporphyrin IX (Proto IX). Protox is the target site of action for several photodynamically active porphyric herbicides of diphenyl ethers, phenyl heterocycles, and heterocyclic carboxamides [2]. The bicyclic nature of diphenyl ethers allows competitive inhibition of Protox located in the plastid by occupying the binding site for Protogen IX [3], so Protogen IX that accumulates in the plastid leaks out and is oxidized to Proto IX by a herbicide-insensitive peroxidase-like enzyme located in the plasma membrane [4]. The resulting Proto IX causes photodynamic membrane peroxidation and, ultimately, cell death in the presence of molecular oxygen and light [5].

Several strategies have evolved for the development of plants that are resistant to porphyric herbicides that target Protox. One approach is the development of transgenic plants that express naturally occurring herbicide-resistant Protox genes. *Bacillus subtilis* Protox is only weakly inhibited by these herbicides [6], and we previously reported that transgenic expression of the *B. subtilis* Protox gene increased resistance to oxyfluorfen in tobacco [7] and rice [8,9] plants. In addition to the naturally occurring Protox genes that are insensitive to herbicide inhibition, screening of herbicide-resistant mutants in Protox inhibitor-containing medium has identified the gene that confers resistance to an alteration of an amino acid residue in the herbicide binding site of the catalytic cleft of Protox. Randolph-Anderson et al. [10] isolated the Val389Met point mutant of *Chlamydomonas reinhardtii* Protox that confers resistance to porphyric herbicides such as S23142, oxadiazon, acifluorfen-ethyl, and oxyfluorfen. The Val389Met substitution in the *C. reinhardtii rs-3* mutant either directly or indirectly causes a change in the conformation of the Protox active site, which decreases its affinity for herbicides. Therefore, the Val389Met mutation in *C. reinhardtii* seems to play an important role in the Protox resistance mechanism. This valine residue is conserved in *Arabidopsis thaliana* Protox, tobacco plastid Protox I, mouse mitochondrial Protox, and *B. subtilis* Protox.

In this paper, we describe the kinetic characterization of a Val311Met mutant of *B. subtilis* Protox. We undertook this study in order to asses the effect of Val311Met substitution of *B. subtilis* Protox on the resistance to oxyfluorfen and possibly improve the resistance of the *B. subtilis* Protox gene to diphenyl ether herbicides, since our previous results [7–9] indicated that the transgenic expression of *B. subtilis* Protox in the cytoplasm or plastid of tobacco and rice plants led to a slightly increased resistance to diphenyl ether herbicides. Our results may provide information for designing Protox genes that interact with diphenyl ether herbicides.

2. Materials and methods

2.1. Chemicals and reagents

Proto IX and Tweeen 20 were obtained from Sigma Chemical (St. Louis, Missouri, USA). DNA QIAEX purification kit was purchased from Qiagen (Hilden, Germany). Restriction enzymes and T4 DNA ligase were obtained from Promega (Madison, Wisconsin, USA). Nickel affinity column was purchased from Invitrogen (NV Leek, The Netherlands). All other chemicals were highest grade available.

2.2. Site-directed mutagenesis

The Val311Met point mutation was introduced into the *B. subtilis* Protox gene using PCR-based site-directed mutagenesis as follows. The *B. subtilis* Protox gene cloned into pRSET B [11] was used as a template. Two sense and two antisense primers were used: the sense primers were 5′-GCCTGCAAGCAAAACAAAGG-3′ (primer A) and 5′-TCCACATCC**A**TGGCAAACGT-3′ (primer B), and the antisense primers were 5′-ACGTTTGCCA**T**GGATGTGGA-3′ (primer C) and 5′-CGACAATGGATTCGTCTCCG-3′ (primer D). The changed nucleotides are written in bold face. PCR was performed using the primer sets A and C or B and D, template DNA, dNTPs, and *Taq* polymerase. The PCR program was as follows: 5 min at 95 °C (1 cycle); 15 s at 95 °C, 15 s at 55 °C, 20 s at 72 °C (15 cycles), and a final extension of 6 min at 72 °C. Two PCR products of 518 and 207 bp were excised from the gel and purified using an agarose gel extraction kit (Qiagen). The purified 518- and 207-bp PCR fragments were then used as templates in a second round of PCR that was performed with primers A and D. The mutated sequence was confirmed by Sanger's dideoxy sequencing method.

2.3. Protein expression and purification

The mutated DNA fragment (706 bp) was digested with *Eco*RI/*Nde*I and ligated into the pRSET B vector, which carries a His-tag sequence. To express recombinant protein [11], the pRSETB(protox) plasmid was transformed into the BL21(DE3)-pLysS strain of *Escherichia coli*. Transformed cells carrying pRSETB(protox) were inoculated into 0.5 L of LB with 100 μg/ml ampicillin and 35 μg/ml chloramphenicol and grown at 25 °C with 200 rpm shaking until an A_{600} of 0.5 was reached. The expression of the recombinant Protox enzyme was induced by adding IPTG (1 mM). Cells were grown for additional 5 h at 25 °C and harvested. The induced recombinant Protox protein was found in inclusion bodies if cells were grown for longer than 5 h or at a temperature higher than 25 °C.

2.4. Purification and assay of Protox enzymes

Cells were harvested by centrifugation, resuspended in 50 mM sodium phosphate buffer, pH 7.4, containing 0.3 M NaCl, 0.5 mM PMSF, 0.2% Tween 20, 1% 1,

10-phenanthroline and disrupted by sonication. Cellular debris was removed by centrifugation at 100,000*g*, the supernatant was collected, and resulting crude extract was subjected to Ni-affinity chromatography. The Ni-column was equilibrated with 10 mM Hepes, pH 7.9, containing 5 mM $MgCl_2$, 0.1 mM EDTA, 50 mM NaCl, 17% glycerol. Crude extract was loaded on the Ni-column, washed, and eluted with equilibration buffer containing gradient of 50–700 mM imidazole. The Protox enzyme eluted at 500 mM imidazole. Protogen IX was prepared *via* reduction of Proto IX with 3% sodium amalgam as previously reported [6]. The Protogen IX solution was adjusted to pH 8.0 by dropwise addition of 40% phosphoric acid and stored in liquid nitrogen. Protox activity was assayed by measuring the rate of appearance of Proto IX, as detected by fluorescence emission at 622 nm with excitation at 395 nm at 25 °C.

2.5. Inhibition kinetics

Oxyfluorfen recrystallized in ethyl alcohol was dissolved in DMSO and used as an inhibitor stock. The enzymatic reaction rate was measured in 100 mM sodium phosphate buffer (pH 7.4) containing 0.1 mM EDTA, 0.1% Tween 20, 5 μM FAD, 2% DMSO, and 500 mM imidazole [12].

3. Results and discussion

3.1. Amino acid sequence comparison of the C. reinhardtii Protox with other Protoxes including B. subtilis Protox

Since the *C. reinhardtii* Protox Val389Met point mutation confers herbicide resistance to the *C. reinhardtii rs-3* mutant, we compared the amino acid sequences of Protox enzymes from *C. reinhardtii* and *B. subtilis* with those of other Protox enzymes known to be sensitive to diphenyl ether herbicides [13]. As shown in Fig. 1, the analysis result indicated that valines equivalent to methionine-389 in *C. reinhardtii rs-3* mutant Protox are conserved in all Protox enzymes sensitive to diphenyl ether herbicides and the methionine-389 in *C. reinhardtii rs-3* mutant corresponds to valine-311 in *B. subtilis*.

Bacillus	306	MHSTSVANVALCFPE GSVQMEH-------E GTGFVISRNS---DFAIT	343
Chlamydomonas	384	FDYPP**M**GAVTLSYPL SAVREERKASDGSVP GFGQLHPRTQ---GITTL	428
Arabidopsis	360	LYYPPVAAVSTSYPK PATRTKC-LTDGELK GFGQLHPRTQ---GVETL	403
Tobacco I	371	FYYPPVGAVTTTYPQ PATRDER-LVDGELK GFGQLHPRTQ---GVETL	414
Tobacco II	324	NFIPEVDYVPLSVVI TTFKREN--VKYPLE GFGVLVPSKEQQHGLKIL	369
Mouse	309	IKAVSVAVVNLQYRG ACLPVQG-------- -FGHLVPSSE---DPTVL	344

Fig. 1. Amino acid sequence alignment of *B. subtilis* (M97208) Protox, *C. reinhardtii rs-3* (AF068635) mutant Protox, *Arabidopsis* Protox (D83139), tobacco Protox I (Y13465), tobacco Protox II (Y13466), Mouse Protox (U25114). Sequences were aligned with ClustalW 1.8 Multiple Sequence Alignments program (BCM Search Launcher). The mutated methionine that corresponds to valine in wild type *C. reinhardtii* at position 389 is shown in bold.

3.2. Mutagenesis and biochemical characterization of mutant Protox enzyme

The Val311Met point mutation was introduced into the *B. subtilis* Protox gene by site-directed mutagenesis as described in Section 2. The mutant Protox enzyme was heterologously expressed in *E. coli* BL21(DE3) cells and purified with a Ni^{2+}–NTA–agarose resin. SDS–PAGE of purified Protox is shown in Fig. 2. The specific activity of mutant Protox was slightly lower than that of wild type Protox; however, the pH dependence of the specific activity (Fig. 3) and the UV-spectra of the purified Protox (data not shown) indicated that the Val311Met point mutation did not significantly alter the stability and/or the overall structural conformation of Protox.

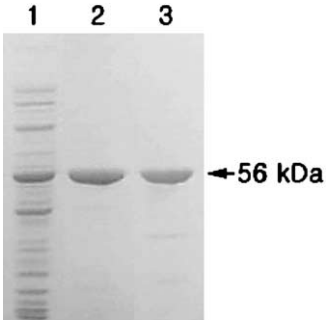

Fig. 2. SDS–PAGE of purified wild type and Val311Met mutant *B. subtilis* Protox. Lane 1: crude extract of Val311Met mutant Protox; lane 2: purified Val311Met mutant Protox; lane 3: purified wild type Protox. Protox was heterologously expressed in *E. coli* and recombinant Protox was purified by Ni-column chromatography as described in Section 2.

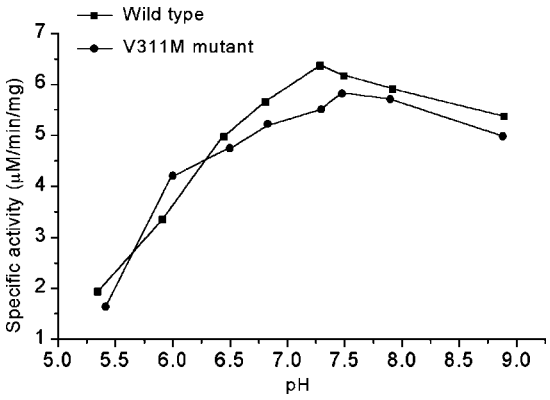

Fig. 3. Dependence of purified *B. subtilis* Protox specific activity on pH. Activities were measured in 100 mM sodium phosphate buffer (pH 7.4) containing 0.1 mM EDTA, 0.1% Tween 20, and 5 μM FAD. (■) Wild type Protox, (●) V311M mutant Protox.

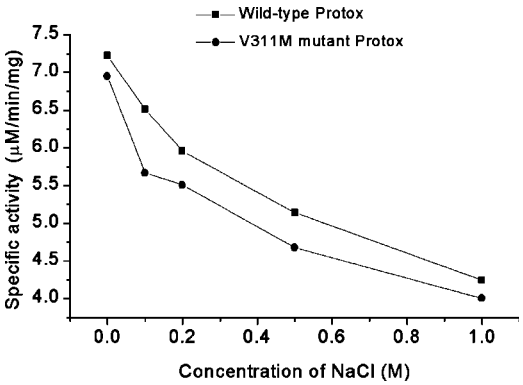

Fig. 4. Dependence of the specific activity of purified *B. subtilis* Protox on NaCl concentration. Activities were measured in 100 mM sodium phosphate buffer (pH 7.4) containing 0.1 mM EDTA, 0.1% Tween 20, and 5 μM FAD. (■) Wild type Protox, (●) V311M mutant Protox.

Since Proto IX is known to be more hydrophobic than Protogen IX [4], the enzymatic oxidation rate of Protogen IX may depend on the hydrophobicity of the reaction medium [14]. As seen in Figs. 4 and 5, the activities of both wild-type and mutant Protox were reduced by NaCl and enhanced by Tween 20 or imidazole. The reduced oxidation rate of Protogen IX in the presence of NaCl might be due to the increased ionic strength of the reaction medium, which is not favorable for the stabilization of the product-like hydrophobic transition state of Protogen IX oxidation. In contrast, Tween 20 and imidazole may contribute to the hydrophobicity of the reaction medium and thereby provide a favorable environment for the oxidation of Protogen IX.

3.3. Kinetics and competitive inhibition by oxyfluorfen

Since Protox activity was enhanced by Tween 20 and imidazole as shown in Figs. 5A and B, all kinetic experiments were carried out in the presence of 0.1% Tween 20 and 500 mM of imidazole. Kinetic constants determined by a Lineweaver–Burk plot in these conditions were $K_{mapp} = 3.2\,\mu M$, $k_{cat} = 14.1\,min^{-1}$ for the wild type Protox, and $K_{mapp} = 3.2\,\mu M$, $k_{cat} = 12.9\,min^{-1}$ for the V311M mutant. This result confirms that the Val311Met point mutation did not cause any significant overall conformational change to the Protox enzyme. When oxyfluorfen was employed as an inhibitor in the Protox-catalyzed oxidation of Protogen IX, the inhibition kinetics indicated that oxyfluorfen acts as a typical competitive inhibitor for both wild type and Val311Met mutant Protox (Fig. 6). The inhibition constants (K_i) were determined to be 11.0 μM (Fig. 6A) for wild type and 17.7 μM (Fig. 6B) for mutant Protox. Therefore, the inhibition constant of Protox was only marginally increased about 1.5 times by the Val311-Met point mutation of *B. subtilis* protoporphyrinogen oxidase. This result

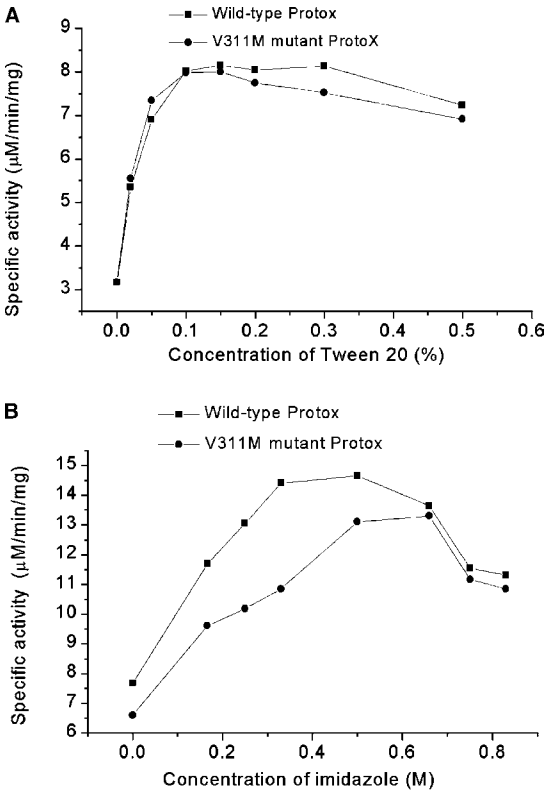

Fig. 5. Dependence of the specific activity of purified *B. subtilis* Protox on concentrations of Tween 20 and imidazole. Activities were measured in 100 mM sodium phosphate buffer (pH 7.4) containing 0.1 mM EDTA, 5 μM FAD, and various concentrations of Tween 20 without imidazole (A) or 0.1% Tween 20 with various concentrations of imidazole (B). (■) Wild type Protox, (●) V311M mutant Protox.

indicates that the Val311Met point mutation in *B. subtilis* protoporphyrinogen oxidase may not cause any significant structural changes at the active site in terms of binding with oxyfluorfen and the mutated methionine residue may not participate in the mechanism of protoporphyrinogen oxidase inhibition by diphenyl ether herbicides. The observed marginal increase of K_i in mutant Protox is probably due to the fact that wild type *B. subtilis* Protox itself is already resistant to diphenyl either herbicides and there might not be any room for further increase of resistance against oxyfluorfen by Val311Met point mutation. More studies must be performed for the characterization of structural determinants responsible for the observed insensitivity of the mutant Protox to oxyfluorfen.

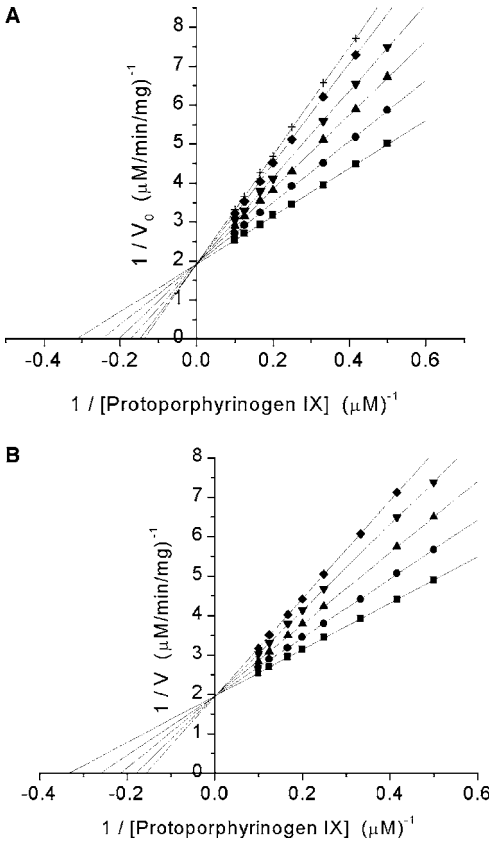

Fig. 6. Inhibition kinetics of purified *B. subtilis* Protox at various oxyfluorfen concentrations. Rates were measured in 100 mM sodium phosphate buffer (pH 7.4) containing 0.1 mM EDTA, 0.1% Tween 20, 5 μM FAD, 2% DMSO, and 500 mM imidazole with various concentrations of oxyfluorfen: 0 μM (■), 3 μM (●), 6 μM (▲), 9 μM (▼), 12 μM (◆), and 15 μM (+) for wild type Protox (A), and 0 μM (■), 5 μM (●), 10 μM (▲), 15 μM (▼), and 20 μM (◆) for the V311M mutant Protox (B).

Acknowledgments

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